VOLUME 29 DECEMBER 1951 NUMBER 6

Canadian Journal of Medical Sciences

Editor: J. B. COLLIP

Published by THE NATIONAL RESEARCH COUNCIL
OTTAWA CANADA

CANADIAN JOURNAL OF MEDICAL SCIENCES

This was formerly *Section E, Canadian Journal of Research.* The change to the new name took place January 1, 1951. The CANADIAN JOURNAL OF MEDICAL SCIENCES is published six times annually.

The CANADIAN JOURNAL OF MEDICAL SCIENCES is published by the National Research Council of Canada under the authority of the Chairman of the Committee of the Privy Council on Scientific and Industrial Research. Matters of general policy are the responsibility of a joint Editorial Board consisting of members of the National Research Council of Canada and the Royal Society of Canada.

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Canadian
Journal of Medical Sciences

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOL. 29 DECEMBER, 1951 NUMBER 6

DIAGNOSTIC CULTURES OF TUBERCLE BACILLI IN MODIFIED DUBOS' MEDIUM[1]

BY JACK KONOWALCHUK[2] AND G. B. REED[3]

Abstract

Comparative series of cultures from tuberculous sputum in Lowenstein's solid and Dubos' fluid medium indicate that growth of tubercle bacilli may be detected much earlier in Dubos' fluid than in any presently known solid medium. A primary difficulty with sputum cultures in Dubos's medium is contamination with nonacid-fast bacteria. It is shown in this paper that contamination may be largely overcome by adding 1–1,000,000 gentian violet to the medium. It is shown that the dye does not inhibit growth of tubercle bacilli. As the preparation of inocula for solid and fluid media is the same it is proposed that diagnostic cultures should be put up in Dubos's gentian violet and conventional solid media from the one preparation. Tentative conclusions may be drawn from microscopic examination of fluid media cultures after 7 to 10 days' incubation and confirmatory evidence obtained from solid media at a later date.

The possibility of using Dubos's and Davis's (1) method of cultivating tubercle bacilli as a diagnostic procedure has been investigated in several laboratories (3, 4, 9). Notwithstanding the fact that tubercle bacilli grow more rapidly in this medium than in any previously used, there are certain disadvantages in its use for diagnostic cultures. In the case of cultures from sputum the greatest difficulty results from the frequent heavy growth of contaminating bacteria. It is generally possible to demonstrate acid-fast bacteria microscopically after five to seven days' incubation of the sputum cultures, even where they are overgrown with contaminants, but such cultures are very unsatisfactory to handle and in those which fail to show acid-fast organisms there is always the possibility that the growing contaminants may have inhibited growth of the tubercle bacilli.

This paper attempts to demonstrate a method by which tubercle bacilli may be cultivated from sputum with little trouble from contaminating organisms and without loss of the chief advantage of Dubos's medium—rapid growth of tubercle bacilli from a small inoculum.

[1] *Manuscript received July 25, 1951.*
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Material and Methods

Samples of sputum were obtained from a tuberculosis sanitarium. In the usual procedure 10 ml. amounts of sputum were mixed with an equal volume of 4% sodium hydroxide and shaken for one hour in a reciprocating shaker operating at 90 strokes per min. The samples were then neutralized with *N* hydrochloric acid with two indicators, phenol red and bromothymol blue papers. Acid was added to the point of the first yellow tinge of the phenol red and first blue tinge of bromothymol blue. After neutralization the samples were centrifuged 15 min. at 4500 r.p.m. and the supernatant discarded. The sediment was suspended in 5 ml. of sterile water and 1 ml. of this suspension used as inoculum for each tube of medium. Assuming that all the tubercle bacilli in the sputum survived the alkali treatment and that all were thrown down in the centrifuging the inoculum is equivalent to 2 ml. of sputum.

The fluid culture medium used in this work was made according to the formula of Dubos and Davis (1) as modified by Dubos and Middlebrook (2) and Pierce, Dubos, and Middlebrook (6). The method of preparation is as follows:

Dissolve in 850 ml. water:

KH_2PO_4	1.0 gm.
Na_2HPO_4	6.5 gm.
Asparagine	2.0 gm.
Tryptic digest of casein (0.1 gm. casein/1 ml. (8))	2.0 ml.

Add from stock solutions:

Ferric ammonium citrate	50 mgm.
$MgSO_4$	10 mgm.
$CaCl_2$	0.5 mgm.
$ZnSO_4$	0.1 mgm.
$CuSO_4$	0.1 mgm.

Add water to make 1000 ml.

Adjust to pH 6.8, dispense in 5 ml. amounts in 6 × 1 in. test tubes, and autoclave at 10 lb. for 20 min.

Add aseptically to each 5 ml. tube from sterile stock solutions:

Bovine albumin fraction V 0.2 ml.
(5% in 2% sodium chloride, Seitz filtered and heated to 56° for 30 min.)

Glucose 0.1 ml.
(25% autoclaved 10 lb. for 20 min.)

Gentian violet* (when required) 0.1 ml.
(1–20,000 in water, autoclaved 10 lb. per 20 min.)

This medium without gentian violet is water clear. In inoculating from pure cultures of tubercle bacilli growth is evident from an increasing diffuse

* *The gentian violet used in all this work was British Drug House Lot 42520.*

clouding of the medium or the formation of ropey aggregates of organisms. But in cultures heavily inoculated with alkali treated sputum the appearance of growth is frequently obscured or confused by the particulate material introduced with the inoculum.

In the following series of cultures, films were made after 7 to 10 days' incubation at 37° C. The cultures were shaken and enough fluid removed with a Pasteur pipette to cover approximately a sq. cm. of slide. The films were air-dried, fixed with heat, and stained by Yegeau and Budd's (10) modification of Ziehl–Neelson's method.

Comparison of Results on Dubos's and Lowenstein's Medium

In a preliminary series of trials 38 samples of sputum from clinical cases of tuberculosis were treated with alkali, neutralized, centrifuged, and the sediment suspended as described above. The same volume of suspended sediment, 1 ml., was used to inoculate tubes of Dubos's medium, as just described but without gentian violet, and Lowenstein's solid medium (5). The cultures on Lowenstein's medium were checked for characteristic colonies after two, four, and six weeks' incubation. The cultures in Dubos's medium were smeared and examined microscopically after 7 and 10 days' incubation. The results, summarized in Table I, indicate that after six weeks' incubation 36 of the 38 Lowenstein cultures had characteristic colonies of tubercle bacilli, at four weeks 32 of 38, at three weeks 29 of 38, and at two weeks 17 of 38 showed growth of tubercle bacilli. In contrast, in the Dubos medium after seven days' incubation acid-fast bacilli were present in 32 of 38 cultures and after 10 days' incubation they were present in 35 of the 38 cultures. In one sample, No. 25, no growth was obtained in Lowenstein's medium but acid-fast bacilli were present after seven days' incubation in Dubos's medium. In contrast, in three samples, Nos. 3, 27, and 36, growth developed on Lowenstein's between the fourth and sixth week but there was no evidence of growth in Dubos's medium in 10 days' incubation.

Contamination

In this series of 38 sputum cultures in Dubos' medium all showed some contamination and most were heavily contaminated. In another series of 40 sputum cultures in Dubos's medium, Table IV, 35 were contaminated, 20 with spore-forming rods, and 15 with Gram-positive cocci. Attempts were therefore made to eliminate nonacid-fast organisms from the cultures.

Influence of Alkali Treatment on Contamination

Sixteen samples of tuberculous sputum were plated on heart infusion agar by the quantitative drop-plate method (Reed and Reed (7)). Counts of bacteria growing on this medium ranged from 3×10^2 to 8×10^6 per ml. of sputum. The same lots of sputum were then treated with an equal volume of 4% sodium hydroxide and shaken in a reciprocating shaking machine. At

TABLE I

COMPARISON OF CULTURES OF TUBERCLE BACILLI FROM ALKALI TREATED SPUTUM ON LOWENSTEIN'S SOLID AND DUBOS'S LIQUID MEDIA

Sample	Lowenstein*				Dubos**	
	2 weeks	3 weeks	4 weeks	6 weeks	7 days	10 days
1	−	−	−	−	+	+
2	+	+	+	+	+	+
3	−	−	−	+	−	−
4	+	+	+	+	+	+
5	+	+	+	+	+	+
6	−	+	+	+	+	+
7	−	+	+	+	+	+
8	−	+	+	+	+	+
9	−	+	+	+	+	+
10	+	+	+	+	+	+
11	−	+	+	+	+	+
12	+	+	+	+	+	+
13	−	+	+	+	+	+
14	−	+	+	+	+	+
15	−	+	+	+	+	+
16	−	+	+	+	+	+
17	−	+	+	+	+	+
18	−	+	+	+	+	+
19	−	−	+	+	+	+
20	+	+	+	+	+	+
21	+	+	+	+	+	+
22	+	+	+	+	+	+
23	−	−	+	+	+	+
24	+	+	+	+	+	+
25	−	−	−	−	+	+
26	−	+	+	+	−	+
27	−	−	−	+	−	−
28	−	−	−	+	+	+
29	+	+	+	+	−	+
30	+	+	+	+	+	+
31	+	+	+	+	−	+
32	+	+	+	+	+	+
33	+	+	+	+	+	+
34	+	+	+	+	+	+
35	+	+	+	+	+	+
36	−	−	−	+	−	−
37	−	−	+	+	+	+
38	+	+	+	+	+	+
Per cent positive	45	76	84	94	84	86

* *Lowenstein* + = *colonies of tubercle bacilli present.*
** *Dubos* + = *tubercle bacilli demonstrated in microscopic preparations.*

intervals of one-half, one, and two hours samples were removed, neutralized, centrifuged, and the sediment suspended in a volume of sterile water equal the original volume of sputum. These suspensions were then plated in the same manner as the untreated sputum; counts of nonacid-fast bacteria ranged from 10,000 to less than 100 per ml. of treated sputum. Less than 100 viable nonacid-fast bacteria were present in 11 of 16 samples after one-half hour of alkali treatment, 13 of 16 after one hour and 15 of 16 samples after two hours

of alkali treatment. However, when these alkali treated samples were introduced into Dubos' medium and incubated for 10 days, heavy contamination developed in 10 of 16 samples after one-half hour, 9 of 16 after one hour and 3 of 16 after two hours of alkali treatment.

Gentian Violet

Several antibiotics and a series of dyes as long used in solid media, together with several other possible substances were added to Dubos's medium which was then inoculated with alkali treated sputum. From this preliminary trial the most promising substances appeared to be gentian violet, basic fuchsin, Congo red, azochloramid, and merthiolate. Some 12 samples of tuberculous sputum were treated with alkali for one hour in the usual manner and equal volumes of resuspended sediments were introduced into a series of tubes of Dubos's medium without added material and to similar Dubos's medium containing the bacteriostatic agents indicated in Table II. It is apparent from the table that samples which gave positive cultures of tubercle bacilli in plain Dubos's also gave positive cultures in Dubos's with gentian violet. But where all the cultures in plain Dubos's medium were heavily contaminated, 3 of 12 in Dubos's with gentian violet were contaminated.

TABLE II

INFLUENCE OF VARIOUS AGENTS ON THE GROWTH OF THE TUBERCLE BACILLI AND CONTAMINATING BACTERIA IN DUBOS'S MEDIUM INOCULATED WITH ALKALI TREATED SPUTUM AND INCUBATED 10 DAYS

Sample	Dubos control		Dubos + 1 : 1,000,000 gentian violet		Dubos + 1 : 1,000,000 basic fuchsin		Dubos + 1 : 1,000,000 Congo red		Dubos + 1 : 1,000,000 azochloramid		Dubos + 1 : 1,000,000 merthiolate	
	T.B.	Contam.	T.B.	Contam.	T.B.	Contam.	T.B.	Contam.	T.B.	Contam.	T.B.	Contam.
1	+++	+++	+++	—	—	—	+	+++	—	—	—	—
2	+++	++	+++	—	—	—	+++	+++	—	—	—	—
3	+++	+	+++	—	—	—	+++	—	—	—	—	—
4	++	+++	+++	—	—	—	+	+++	—	—	—	—
5	—	+++	—	—	—	—	—	—	—	+++	—	—
6	—	++	—	—	—	—	—	—	—	+++	—	—
7	—	++	—	—	—	—	—	—	—	++	—	—
8	++	++	+	—	—	—	—	—	++	+++	—	—
9	+++	+++	+++	—	+++	—	—	—	—	—	++	+++
10	+	+++	+	++	—	+++	—	—	—	—	—	++
11	+	++	+	++	+	++	—	—	—	—	—	++
12	+++	++	+++	+	+++	+++	—	—	—	—	++	++

NOTE: *Contaminants:* + = *Few per field.*
++ = *Many per field.*
+++ = *Field covered.*
Tubercle bacilli: + = *Occasional organism per field.*
++ = *Several per field.*
+++ = *Many per field.*

The other bacteriostatic agents listed in Table II while equally effective in reducing contamination also reduced or inhibited growth of tubercle bacilli.

In a further trial of the influence of gentian violet on the growth of tubercle bacilli from sputum in Dubos's medium seven samples were treated with alkali for one hour in the usual manner and inoculated into plain Dubos's and Dubos with 1–1,000,000 and 1–2,000,000 concentrations of gentian violet. At the end of 7 and 10 days' incubation smears were made and the amount of growth in the three lots of media compared, Table III. It is apparent from the table that growth of tubercle bacilli was similar in the three series of media. In so far as the method of examination permitted, an estimate of the amount of growth showed it to be slightly heavier in the media containing gentian violet. This probably results from the inhibition of contamination.

TABLE III

GROWTH OF TUBERCLE BACILLI FROM SPUTUM TREATED ONE HOUR WITH ALKALI IN DUBOS'S MEDIUM AND IN DUBOS'S WITH 1–1,000,000 AND 1–2,000,000 CONCENTRATION OF GENTIAN VIOLET

Sample	Dubos's medium		Dubos's+1–1,000,000 gentian violet		Dubos's+1–2,000,000 gentian violet	
	7 day	10 day	7 day	10 day	7 day	10 day
1	+	+	++	++	++	++
2	++	++	++	++	++	++
3	—	+	—	+	—	+
4	+++	+++	++	++	++	++
5	++	++	++	++	++	++
6	+	+	+	+	++	++
7	++	++	++	++	++	++
Total positive	6	7	6	7	6	7
Number of +'s	11	12	11	12	12	13

NOTE: + = *Occasional acid-fast organisms.*
++ = *Several acid-fast organisms per field.*
+++ = *Many acid-fast organisms per field.*

In a further series 40 samples of sputum were treated with alkali for one hour, neutralized, centrifuged, and the same volume of sediment introduced into plain Dubos's medium and similar Dubos's containing 1–1,000,000 concentration of gentian violet. The results after 10 days' incubation are summarized in Table IV. As shown in the table, 35 of 40 cultures in Dubos's medium were contaminated with spore-forming rods or Gram-positive cocci and in 33 of the 35 there was a heavy growth of the contaminating species. In contrast, in Dubos's medium with gentian violet inoculated with the same alkali treated sputum 7 of 40 cultures were contaminated, five with slight and two with heavy growths.

It is also indicated in Table IV that 20 of 40 cultures in plain Dubos's medium developed tubercle bacilli which could be demonstrated microscopically notwithstanding the contamination. But in the Dubos's medium with

TABLE IV

GROWTH OF TUBERCLE BACILLI AND CONTAMINATING NONACID-FAST BACTERIA IN DUBOS'S MEDIUM, WITH AND WITHOUT GENTIAN VIOLET, AFTER 10 DAYS' INCUBATION AFTER INOCULATION WITH SPUTUM TREATED WITH ALKALI FOR ONE HOUR

Sample	Dubos's medium		Dubos's + 1 : 1,000,000 gentian violet	
	Contaminants	Tubercle bacilli	Contaminants	Tubercle bacilli
1	C +	++	—	++
2	R +++	+	—	+
3	—	—	—	—
4	—	—	—	—
5	C ++	—	—	—
6	R +++	—	—	+
7	C ++	++	—	++
8	C ++	++	—	++
9	C ++	+	—	+
10	C ++	—	C ++	—
11	R +++	—	—	—
12	C +	++	—	++
13	R +++	+	C +	+
14	R +++	—	—	—
15	C ++	—	—	—
16	C ++	+++	—	+++
17	C +	++	C +	++
18	R +++	++	R +	++
19	R +++	—	C +	++
20	C +	—	R +	+
21	—	+	—	+
22	R ++	++	—	+++
23	R +++	—	—	—
24	—	—	—	—
25	C ++	+	—	++
26	R ++	—	—	—
27	R ++	—	—	—
28	R ++	—	—	—
29	C ++	—	—	—
30	R ++	—	—	—
31	C ++	—	—	—
32	R ++	—	—	—
33	C ++	++	—	+
34	R ++	+	—	++
35	—	+++	—	+++
36	R ++	—	—	+
37	R ++	+++	—	+++
38	R ++	+	—	+++
39	R ++	+++	R ++	+++
40	R ++	++	—	+++
	87%	50%+	17%	60%

NOTE: *Contaminants:* *R* = *Spore-forming rods.*
C = *Gram + cocci.*
\+ = *Few per field.*
++ = *Many.*
+++ = *Very heavy growth.*
Tubercle bacilli: + = *Occasional organism.*
++ = *Several per field.*
+++ = *Many per field.*

gentian violet 24 of 40 cultures developed tubercle bacilli, i.e., four sputa which gave negative cultures in plain Dubos's medium developed tubercle bacilli in the Dubos's medium with gentian violet. Moreover, for the most part, the growth of tubercle bacilli in the gentian violet containing medium was more luxuriant than in the medium without the dye. This may be due either to the production of toxic substances by the massive growth of the contaminating organisms or merely results from exhaustion of the medium by the contaminants.

Discussion

It is apparent that contamination of sputum cultures in Dubos' medium may be controlled to a reasonable degree by the addition of gentian violet without impairing the growth of tubercle bacilli. It is also apparent that by this means positive cultures of tubercle bacilli may be obtained in an appreciably shorter period than with the more conventional procedure of cultivation on solid media.

Solid media cultures give so much more readily readable and realistic results than microscopic demonstration of acid-fast organisms growing in fluid that there is no suggestion that, for the present, fluid media cultures should be adopted for the diagnosis of tuberculosis. On the other hand there is ample evidence to suggest the advisability of adopting the fluid media procedure as a supplement to cultures on solid media. As the preparation of sputum, or other material, for the inoculation of fluid and solid media is the same, little extra labor is involved in putting up solid and liquid media cultures at the same time. It is therefore suggested that when diagnostic cultures are to be made, one lot of inoculum be prepared and that both solid and fluid media be inoculated from the one preparation. After 7 or 10 days' incubation the cultures in Dubos's gentian violet medium may be examined microscopically and tentative conclusions drawn from the findings. The solid media cultures will then be available for confirmatory evidence at a later date.

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CONTENTS OF VOLUME 29

ASHESHOV, I. N. and HEAGY, F. C.—A "loop" method for counting viable bacteria or bacteriophage, 1.

BARBER, V. T.—See Cullen, A. M.

BERGSAGEL, D. E.—See Blanchaer, M. C.

BIRMINGHAM, M. K.—See Cullen, A. M.

BLANCHAER, M. C., WEISS, P., and BERGSAGEL, D. E.—The lactic dehydrogenase activity and pyridine nucleotide content of human blood cells in anemia, 108.*

BOULANGER, P.—See Rice, C. E.

BOULANGER, P. and RICE, C. E.—Parallel studies of complement and coagulation. II. The relative effects of dicumarol, 5.

CLARK, E. M. and RHODES, A. J.—Poliomyelitis in Canadian Eskimos. Laboratory studies. II, 216.

COLEMAN, I. W.—A study, by means of paper chromatography of the growth stimulating properties of liver extract, yeast solubles, and trypsin digest of casein for *Lactobacillus casei*, 151.

CULLEN, A. M., BARBER, V. T., BIRMINGHAM, M. K., and STERN, K.—Peptidase activity in human serum under various pathological conditions, in pregnancy, and following the administration of ACTH, 114.

DAVEY, P. W.—See Howatt, F. F.

DENSTEDT, O. F.—See Saffran, M.

DESMARAIS, A. and DUGAL, L.-P.—Circulation périphérique et teneur des surrénales en adrénaline et en artérénol (Noradrénaline) chez le rat blanc exposé au froid, 90. Influence de l'administration d'adrénaline et d'artérénol sur l'hypertrophie de la surrénale au froid, 104.

DUGAL, L.-P.—Effects of cold, ascorbic acid, and age on "formaldehyde-induced" arthritis in the white rat, 35. See DesMarais, A.; Héroux, O.

DUNLOP, D. L.—See Hildes, J. A.

FORTIER, C.—See Skelton, F. R.

FRENCH, R. C.—See Lesley, S. M.

FRENCH, R. C., LESLEY, S. M., GRAHAM, A. F., and VAN ROOYEN, C. E.—Studies on the relationship between virus and host cell. III. The breakdown of P^{32} labelled T2r^+ bacteriophage adsorbed to *E. coli* previously infected by other coliphages of the T group, 144.

GEMMELL, J. P.—See Perry, W. F.

GRAHAM, A. F.—See French, R. C.; Lesley, S. M.

HEAGY, F. C.—See Asheshov, I. N.

HÉROUX, O. and DUGAL, L.-P.—Effet de l'acide ascorbique sur l'hypertension expérimentale, 164.

HILDES, J. A. and DUNLOP, D. L.—A method for estimating the rates of gastric secretion and emptying, 83.

HOARE, R.—See Tuba, J.

HOWATT, F. F. and DAVEY, P. W.—The effect of ascorbic acid on experimental nephritis, 124.

HUNTER, J. and WHILLANS, M. G.—A study of the effect of cold on joint temperature and mobility, 255.

HUSTON, M. J.—See Riedel, B. E.

JOHNS, C. K.—A "loop" method for counting viable bacteria or bacteriophage, 236.

KONOWALCHUK, J. and REED, G. B.—Diagnostic cultures of tubercle bacilli in modified Dubos' medium, 263.

LESLEY, S. M.—See French, R. C.

LESLEY, S. M., FRENCH, R. C., GRAHAM, A. F., and VAN ROOYEN, C. E.—Studies on the relationship between virus and host cell. II. The breakdown of T2r^+ bacteriophage upon infection of its host *Escherichia coli*, 128.

MCKERNS, K. W.—Paper chromatography of casein and rennin, 59.

MCNABB, A. R.—Enzymes of gray matter and white matter of dog brain. The distribution of certain nonoxidative enzymes, 208.

MEYERHOF, O.—Mechanisms of glycolysis and fermentation, 63.*

* *See Additions and Corrections, p. 272.*

MICHAUD, L.—See Pagé, E.

NADLER, N. J.—Some theoretical aspects of radioautography, 182.

PAGÉ, E. and MICHAUD, L.—The titrimetric determination of plasma fatty acids, 239.

PERRY, W. F. and GEMMELL, J. P.—A note on the postoperative increase in iodine excretion, 149.

PLUMMER, P. J. G.—See Rice, C. E.

REED, G. B.—See Konowalchuk, J.

RHODES, A. J.—See Clark, E. M.

RICE, C. E.—See Boulanger, P.

RICE, C. E., BOULANGER, P., and PLUMMER, P. J. G.—Parallel studies of complement and coagulation. III. The effect of the protein level of the diet, 13. Parallel studies of complement and coagulation. IV. Effect of carbon tetrachloride, 48.

RIEDEL, B. E. and HUSTON, M. J.—Effect of high doses of amytal on skeletal muscle, 79. Effect of sodium kemithal, trimethadione, and sodium diphenylhydantoinate on skeletal muscle, 159.

ROSSITER, R. J.—See Wong, E.

SAFFRAN, M. and DENSTEDT, O. F.—The effect of intravenously injected citrate on the serum ionized calcium in the rabbit, 245.

SKELTON, F. R. and FORTIER, C.—The influence of oral ascorbic acid administration on the adrenal cortical response to ACTH, 100. Plasma ascorbic acid and pituitary–adrenal activity, 176.

STERN, K.—See Cullen, A. M.

TUBA, J. and HOARE, R.—On rat serum lipase. II. The effect of various experimental states, 25.

VAN ROOYEN, C. E.—See French, R. C.; Lesley, S. M.

WEISS, P.—See Blanchaer, M. C.

WHILLANS, M. G.—See Hunter, J.

WONG, E. and ROSSITER, R. J.—Inhibitors of the β-glucuronidase of rabbit polymorphonuclear leucocytes, 195.*

ADDITIONS AND CORRECTIONS

Page 77. The following acknowledgment should appear above the heading **References**.

Acknowledgment

Fig. 1 has been reproduced by courtesy of the Journal of Biological Chemistry and Figs. 2–6 by courtesy of The Archives of Biochemistry.

Page 111. In the last line, Hr should read Hb.

Page 201. In the last line of the caption for Fig. 2, m-mol. should read gm-mol.

* *See Additions and Corrections.*

CANADIAN JOURNAL OF MEDICAL SCIENCES

Notes to Contributors

Contents

Page

Diagnostic Cultures of Tubercle Bacilli in Modified Dubos' Medium—
Jack Kwantes and A. H. Kidd 265

Contents of Volume 29 271